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Publication number: **0 382 120 B1**

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EUROPEAN PATENT SPECIFICATION

43 Date of publication of patent specification: 24.05.95 51 Int. Cl.⁶: C12P 19/04, C12N 1/20

21 Application number: 90102131.1

22 Date of filing: 03.02.90

The file contains technical information submitted
after the application was filed and not included in
this specification

54 Method for producing biologically active polysaccharide ron substance.

30 Priority: 08.02.89 JP 29381/89

43 Date of publication of application:
16.08.90 Bulletin 90/33

45 Publication of the grant of the patent:
24.05.95 Bulletin 95/21

64 Designated Contracting States:
CH DE ES FR GB IT LI

56 References cited:
EP-A- 0 087 404
EP-A- 0 172 559
EP-A- 0 363 633
DE-A- 3 224 547
US-A- 2 823 128

CHEMICAL ABSTRACTS, vol. 110, no. 1, Jan-
uary 2, 1989, Columbus, OH (US); T. SUGURU
et al., p. 392, no. 387w/

CHEMICAL ABSTRACTS, vol. 79, no. 17, Oc-
tober 29, 1973, Columbus, OH (US); L. OLLE,
p. 142, no. 101749n/

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EP 0 382 120 B1

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SNEATH et al., "Bergey's Manual of Systematic Bacteriology", vol. 2, 1986, Williams & Wilkins, Baltimore, MD (US); pp. 1071-1075/

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Description**Background of the Invention**

5 1. Field of the invention

The present invention relates to the preparation of a biologically active polysaccharide RON substance (hereinafter referred to as "RON substance"), in particular to the use of specific microorganism strains for producing physiologically active RON substance having an excellent antitumor activity, an immunomodulating activity and a host defense activity against infectious diseases, which comprises using a specific microorganism strain having an activity of producing this biologically active polysaccharide RON substance or a processed culture broth of this microorganism, and also to novel microorganisms having an activity of producing an excellent biologically active polysaccharide RON substance.

15 2) Description of the Related Art

The RON substance intended in the present invention and a process for extracting this RON substance from rice bran have already been disclosed in JP-B-62-7173.

According to this conventional process, the RON substance is extracted from rice bran and the extract is purified. Therefore, the quality of the starting material is unstable, and there are considerable dispersions of the physical properties and biological activities of the obtained substance. Moreover, the yield is low and many steps are required, and the conventional process involves the problem of long operation time and is economically disadvantageous.

Processes for preparing water-soluble dextrans suitable for clinical use and having a molecular weight between 5000 and 50×10^6 starting from a sucrose-containing substrate are already known from EP-A-0 087 404, from "Chem. Abstracts", Vol. 79, No. 17, page 142, Abstract No. 101 749 n (29.10.73) and from US-A-2 823 128.

These dextrans which are predominantly used as synthetic blood plasma may be obtained by inoculating a nutrient medium containing sucrose and other ingredients with an appropriate microorganism, for example with *Leuconostoc mesenteroides* NRRL B512. However, biologically active polysaccharide RON substance having the above useful activities cannot be obtained by means of these fermentative or enzymatical processes of the prior art.

The same applies to DE-A-32 24 547 which relates to a process for the production of biopolymers by aerobic culturing of *Leuconostoc mesenteroides* DSM 20240 in a water-in-oil emulsion stable under fermentation conditions, and to EP-A-0 363 633 (prior art according to Article 54(3) EPC) which relates to a novel dextran produced by *Leuconostoc dextranicum* NRRL B 18242 containing 1,6-glycopyranose with branching about every 20 to 30 residues of glucose and having about 5 to 20 % by weight of fructose.

The object of the present invention is to find a way to produce RON substance by a microbiological process which provides the RON substance in a stable and high quality.

40 **Summary of the Invention**

Under this background, we made an extensive investigation with a view to overcome the above-mentioned defects of the conventional techniques, and as the result, it was found a process for producing the RON substance with high efficiency when using specific microorganisms. Based on these findings, the present invention has been accomplished.

According to a first aspect subject-matter of the present invention is the use of a microorganism strain selected from the group consisting of *Leuconostoc mesenteroides* subsp. *dextranicum*, strain BL-75 (FERM BP-2242), strain NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 and IFO 3349, and mutants thereof,

for producing in a culture medium containing sucrose as a source of carbon physiologically active polysaccharide RON substance having the following properties:

- (1) Form: the substance is a white amorphous powder having no taste and no smell;
- (2) Solubility: the substance is soluble in water, but the solution becomes milky white and viscous when the concentration is elevated, the substance is soluble in formamide and dimethylsulfoxide, but the substance is insoluble in alcohols, acetone, benzene, ethyl acetate, hexane, chloroform and carbon tetrachloride;

- (3) pH of Aqueous Solution: the aqueous solution is neutral or weakly acidic;
- (4) Constituent Saccharide: the substance is composed solely of glucose;
- (5) Elementary Analysis Values: the substance comprises 44.0 to 45.0 % of C and 6.1 to 6.3 % of H;
- (6) Structure: the substance is an α -1,6-glucan and contains a small amount of 3,6-branched side chains;
- (7) Proteins: the substance contains almost no proteins;
- (8) Molecular Weight: the substance does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
- (9) Specific Rotations: $[\alpha]_D^{25} = +190^\circ \approx +220^\circ$ (C=0.5, formamide);
- (10) Color Reaction: the substance is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (11) Melting Point: the substance has no definite melting point;
- (12) Ultraviolet Absorption Spectrum: the substance has no characteristic absorption;
- (13) Infrared Absorption spectrum; the substance shows a characteristic absorption of α -glucan;
- (14) ^{13}C -NMR Spectrum: the substance shows absorption characteristic chemical shift values of α -1,6-glucan in the main signals; and
- (15) Biological Activity: the substance has an antitumor activity.

According to a second aspect of the present invention there is provided a process for the production of biologically active polysaccharide RON substance which comprises culturing a microorganism strain selected from the group consisting of *Leuconostoc mesenteroides* subsp. *dextranicum*, strain BL-75 (FERM BP-2242), strain NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 and IFO 3349, and mutants thereof,

for producing in a culture medium containing sucrose as a source of carbon physiologically active polysaccharide RON substance having the following properties, and collecting the physiologically active polysaccharide RON substance from the culture broth:

- (1) Form: the substance is a white amorphous powder having no taste and no smell;
- (2) Solubility: the substance is soluble in water, but the solution becomes milky white and viscous when the concentration is elevated, the substance is soluble in formamide and dimethylsulfoxide, but the substance is insoluble in alcohols, acetone, benzene, ethyl acetate, hexane, chloroform and carbon tetrachloride;
- (3) pH of Aqueous Solution: the aqueous solution is neutral or weakly acidic;
- (4) Constituent Saccharide: the substance is composed solely of glucose;
- (5) Elementary Analysis Values: the substance comprises 44.0 to 45.0 % of C and 6.1 to 6.3 % of H;
- (6) Structure: the substance is an α -1,6-glucan and contains a small amount of 3,6-branched side chains;
- (7) Proteins: the substance contains almost no proteins;
- (8) Molecular Weight: the substance does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
- (9) Specific Rotation: $[\alpha]_D^{25} = +190^\circ \approx +220^\circ$ (C=0.5, formamide);
- (10) Color Reaction: the substance is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (11) Melting Point: the substance has no definite melting point;
- (12) Ultraviolet Absorption Spectrum: the substance has no characteristic absorption;
- (13) Infrared Absorption Spectrum; the substance shows a characteristic absorption of α -glucan;
- (14) ^{13}C -NMR Spectrum: the substance shows absorption characteristic chemical shift values of α -1,6-glucan in the main signals; and
- (15) Biological Activity: the substance has an antitumor activity.

According to a preferred embodiment of the invention it relates to a process, wherein as the microorganism strain *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) or *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670) is used.

According to a third aspect subject-matter of the present invention is a biologically pure culture of the microorganisms *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) and *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670).

Brief Description of the Drawings

Fig. 1 shows the ultraviolet absorption spectrum of the RON substance obtained by the present invention.

5 Fig. 2 shows the infrared absorption spectrum of the RON substance.

Fig. 3 shows the ^{13}C -NMR spectrum of the RON substance.

Detailed Description of Preferred Embodiments

10 According to the present invention, the RON substance can be obtained by using specific microorganisms as stated above belonging to the genus *Leuconostoc* and having an ability of producing the RON substance in a culture medium to accumulate the RON substance in the culture broth and then collecting said RON substance. The RON substance can also be obtained by treating a processed culture broth of these microorganisms with sucrose to form the biologically active polysaccharide RON substance and then
15 collecting the RON substance.

As specific examples of the microorganisms capable of producing the RON substance there can be mentioned *Leuconostoc mesenteroides* subsp. *dextranicum*, strain BL-75 (FERM BP-2242) and strain 46-1 (FERM BP-2670) isolated from rice bran. The microbiological characteristics of these strains are as follows.

20 Strain BL-75 (FERM BP-2242)

| | | |
|----|--|--|
| | Gram stain: | + |
| | Morphological characteristics: | spherical to ellipsoidal, 0.4 to 0.7 μm , chain of 3 to 4 cocci, formation of cluster |
| 25 | Catalase reaction: | - |
| | Oxidase reaction: | - |
| | Free oxygen demand: | facultative anaerobic |
| | Decomposition of arginine: | - |
| | Lactic acid fermentation: | hetero type, D-lactic acid |
| 30 | Production of acid from carbohydrates: | |
| | | arabinose: - |
| | | fructose: + |
| | | galactose: + |
| | | glucose: + |
| 35 | | lactose: + |
| | | mannose: + |
| | | trehalose: + |
| | Hydrolysis of esculin: | + |
| | Production of dextran: | + |
| 40 | Growth in the presence of NaCl: | |
| | | 3.0% NaCl: + |
| | | 6.5% NaCl: - |
| | Growth at different initial pH values: | |
| | | pH 4.8: - |
| 45 | | pH 6.5: + |
| | Final pH in glucose medium: | 4.3 |

Strain 46-1 (FERM BP-2670)

| | | |
|----|--|---|
| 50 | Gram stain: | + |
| | Morphological characteristics: | spherical to ellipsoidal, 0.4 to 0.6 μm , pair and short chain |
| | Catalase reaction: | - |
| | Oxidase reaction: | - |
| | Free oxygen demand: | facultative anaerobic |
| 55 | Decomposition of arginine: | - |
| | Lactic acid fermentation: | hetero type, D-lactic acid |
| | Production of acid from carbohydrates: | |
| | | arabinose: - |

| | |
|----|--|
| | fructose: + |
| | galactose: + |
| | glucose: + |
| | lactose: + |
| 5 | mannose: + |
| | trehalose: + |
| | Hydrolysis of esculin: + |
| | Production of dextran: + |
| | Growth in the presence of NaCl: |
| 10 | 3.0% NaCl: + |
| | 6.5% NaCl: - |
| | Growth at different initial pH values: |
| | pH 4.8: ± |
| | pH 6.5: + |
| 15 | Final pH in glucose medium: 4.1 |

In view of the foregoing, the mycological properties of both strains are summarized as follows.

1. Both strains are positive to the Gram stain and are facultative anaerobic.
2. The morphological characteristics is in a chain of spherical to ellipsoidal cocci.
3. Both strains are positive to the production of acid from carbohydrates except arabinose.
- 20 4. The lactic acid fermentation is of the hetero type, and only the D-form of lactic acid is formed.
5. Both strains are negative to the decomposition of arginine.

With reference to Bergey's Manual of Determinative Bacteriology, 8th edition, it was judged that the strain BL-75 belonged to the genus Leuconostoc, and since the strain was positive to the production of dextran and negative to the production of an acid from arabinose and the strain did not grow in the presence of 6.5% NaCl, therefore, the strain BL-75 was identified as a strain of Leuconostoc dextranicum and deposited as FERM BP-2242 in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry.

Afterward, we learned by reading after described new handbooks that the name of the species of Leuconostoc dextranicum was changed. Accordingly, the strain BL-75 and the strain 46-1 found afterward were investigated again with reference to Bergey's Manual of Systematic Bacteriology, Vol. 2, 9th edition- (1986) and Methods in Microbiology, Vol. 16, pages 147 and 148 (1984). As the result, both the strains were identified as the strains of Leuconostoc mesenteroides subsp. dextranicum. Accordingly, we changed the name of strain BL-75 to Leuconostoc mesenteroides subsp. dextranicum, BL-75, and strain 46-1 was named Leuconostoc mesenteroides subsp. dextranicum, 46-1 and the latter was also deposited as FERM BP-2670 with Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry.

We further searched for the RON substance-producing microorganisms, and it was found that some known strains had an activity of producing the RON substance as well as the strains BL-75 and 46-1. Namely, Leuconostoc mesenteroides subsp. dextranicum, strain NCFB 517 (FERM BP-2711), strain NCFB 531 (FERM BP-2712), strain NCFB 861 (FERM BP-2713), strain NCFB 864 (FERM BP-2714), strain NCFB 880 (FERM BP-2715), strain ATCC 1956 and strain IFO 3349 were found to be the RON substance producers.

Culture of the microorganism capable of producing the RON substance is, in principle, carried out according to an ordinary microorganism-culture method. Since the specific microorganism belonging to the genus Leuconostoc is facultative anaerobic and it demands no oxygen, stationary culture using a liquid medium or gently agitating culture to unify the temperature distribution is generally advantageously adopted.

In the case of obtaining the RON substance directly by using a method of direct culturing, in the culture medium used for culturing, sucrose should be indispensably contained as the carbon source for producing the RON substance. Any of synthetic culture media, semi-synthetic culture media and natural media can be used, so far as other carbon source, a nitrogen source, an inorganic substance and other nutrient sources that can be utilized by the above-mentioned microorganism are contained in addition to sucrose. Any of crude and refined products of sucrose can be optionally used. For example, first-class white refined sugar, muscovado, theriac, blackstrap molasses or sucrose of the reagent grade can be used. A sucrose concentration of about 0.5 to about 70%, preferably 5 to 50% is preferable. As the nitrogen source, there can be used yeast extract, peptone, gluten meal, soybean powder, corn steep liquor, dry yeast, meat extract, ammonium sulfate and urea singly or mixture thereof to the medium in an amount of 0.5 to 5%, preferably 1 to 3%. As the inorganic substance, there can be used phosphoric acid salts and salts of metals

such as magnesium, manganese, iron, cobalt and sodium can be appropriately added.

The incubation temperature may be one for mesophiles, therefore, it is generally 15 to 45 °C, preferably 20 to 30 °C. The pH in culturing is 5 to 7. The incubation time is generally 5 to 96 hours, preferably 10 to 24 hours. The RON substance can be produced in the medium as described above.

5 In the case where the RON substance is obtained by using the processed culture broth of the microorganism, in order to accumulate the RON substance in the above mentioned culture broth, it is indispensable that sucrose should be contained as the carbon source in the culture medium. It is preferred that the sucrose concentration is 0.1 to 10%, especially 1 to 5%. Other conditions such as the culturing pH value, the culturing temperature and the culturing time are the same as those described above with respect
10 to the direct preparation of the RON substance by the culturing of the microorganism.

As the processed culture broth of the microorganism, there can be mentioned a supernatant obtained by subjecting a culture broth of a microorganism having an activity of producing the RON substance to centrifugal separation or the like, a product obtained by pulverizing cells of the culture broth by ultrasonic pulverizer, if necessary removing insoluble substances by centrifugal separation or the like, and a product
15 obtained by dialyzing the above-mentioned processed culture broth at 0 to 30 °C for 5 to 72 hours against a buffer solution having a pH value of 5 to 7, by using a dialysis membrane. The RON substance can be formed by reacting the above-mentioned processed culture broth with sucrose. The reaction temperature is 20 to 45 °C, preferably 25 to 35 °C, and the reaction time is 5 to 50 hours and the pH value is 5 to 7.

The produced RON substance is usually contained in the culture broth or reaction mixture. After the
20 cells and insoluble substances are moved by centrifugal separation, filtration or the like, the RON substance can be collected by carrying out precipitation with a polar organic solvent such as methanol, ethanol, propanol, butanol or acetone and/or salting-out with ammonium sulfate, or repeating these operations. Furthermore, dialysis, gel filtration, ultrafiltration and a treatment with an ion exchange resin or active carbon are carried out singly or in combination, whereby the RON substance having an enhanced purity can be
25 obtained. Moreover, a white powder of the RON substance can be obtained by a spray drying, freeze drying or precipitation with a polar organic solvent and subsequent drying. Still further, the RON substance can be prepared more efficiently by using a bioreactor involving the above-mentioned microorganisms.

The properties of the so-obtained RON substance are as follows.

- (1) Form: the substance is a white amorphous powder having no taste and no smell;
 - 30 (2) Solubility: the substance is soluble in water but the solution becomes milky white and viscous when the concentration is elevated, the substance is soluble in formamide and dimethylsulfoxide but the substance is insoluble in alcohols, acetone, benzene, ethyl acetate, hexane, chloroform and carbon tetrachloride;
 - (3) pH of Aqueous Solution: the 1% aqueous solution is neutral or weakly acidic;
 - 35 (4) Constituent Saccharide: the substance is composed solely of glucose as described below.
- When the present substance was completely hydrolyzed with sulfuric acid and formic acid and the hydrolysis product was analyzed by the thin layer chromatography and high-performance liquid chromatography under conditions described below, a saccharide other than glucose could not be confirmed.

- 40 (1) Thin layer chromatography
Carrier: Silica Gel HP-K® supplied by Wattman
Developing solvent: butanol / acetic acid/water (2/1/1)
- (2) High-performance liquid chromatography
Column: YMC PA-03® supplied by Yamamura Kagaku
- 45 Developing solvent: water/acetonitrile (30/70)

Accordingly, it is obvious that the present substance is a polysaccharide comprising only glucose as the constituent saccharide.

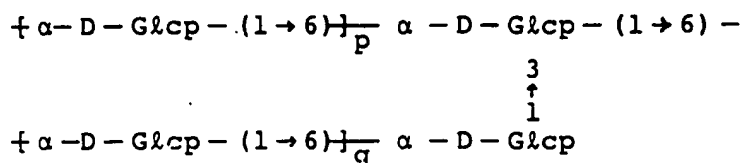
- (5) Elementary Analysis Values: the substance comprises 44.0 to 45.0% of C, 6.1 to 6.3% of H and 0.1% of ash.

- 50 (6) Structure:

The present substance shows characteristic absorptions in the ultraviolet absorption spectrum as shown in Fig. 1, and the present substance shows characteristic absorptions of α -glucan in the infrared absorption spectrum as shown in Fig. 2. Moreover, the present substance shows the characteristic peak of α -1,6-glucan as the main signals in the ^{13}C -NMR spectrum as shown in Fig. 3.

- 55 From these data mentioned above, it is confirmed that the present substance is an α -glucan. Moreover, in the periodic acid oxidation test, the present substance consumes about 1.9 moles of periodic acid per glucose residue to form about 0.98 mole of formic acid, and in the Smith degradation, a large quantity of glycerol is detected. Accordingly, it is presumed that the present substance has a large amount of 1 \rightarrow 6

glucosidic bonds. In order to know a more detailed structure, the present substance was completely methylated and hydrolyzed, and the obtained methyl saccharide was analyzed. As the result, a large amount of 2,3,4-tri-*o*-methylglucose, a small amount of 2,3,4,6-tetra-*o*-methylglucose and 2,4-di-*o*-methylglucose were found. Accordingly, it is presumed that the RON substance obtained by the process of the present invention is an α -1,6-glucan, having a small amount of 3,6-branched side chains and containing glucose as the sole constituent saccharide, and the present substance has the following skeleton:



wherein Glcp represents glucopyranose, and p and q are numbers of from 0 to 50, with the proviso that the requirement of $p + q \leq 50$ is satisfied.

Proteins: the substance contains almost no proteins:

Molecular Weight: the substance does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;

Specific Rotation: $[\alpha]_D^{25} = +190^\circ \sim +220^\circ$ (C=0.5, formamide);

Color Reaction: the substance is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction but negative to biurette reaction, the Lowry--Folin reaction, the Elson-Morgan reaction and iodine reaction:

25 Melting Point: the substance has no definite melting point, and the present substance is browned at 220 °C and blackened at 280 °C to cause carbonization.

Ultraviolet Absorption Spectrum: the substance has no characteristic absorption as shown in Fig. 1;

Infrared Absorption Spectrum: the substance shows a characteristic absorption of α -glucan as shown in Fig. 2:

30 ¹³C-NMR Spectrum: the substance shows characteristic chemical shift values of α-1,6-glucan in the main signals as shown in Fig. 3; and

Biological Activity: the substance has an antitumor activity.

The RON substance obtained by the present invention can be converted to a substance having a lower molecular weight by adding several percents of sulfuric acid, hydrochloric acid or formic acid to the present substance and gently heating the mixture to effect partial hydrolysis. The molecular weight of the molecular weight-reduced RON substance can be unified by gel filtration using Sepharose®, Sephadex® or Tovopearl® or ultrafiltration having various exclusion limits after the neutralization.

It was found that the RON substance and the low-molecular-weight product of the RON substance having a molecular weight higher than about 10000 obtained according to the present invention had various biological activities such as an antitumor activity, an immunomodulating activity and a host defense activity against infectious diseases. The methods for testing these biological activities and the results obtained when the RON substance obtained in Example 1, the low-molecular-weight product of the RON F₁ (having a molecular weight higher than 1,000,000), F₂ (having a molecular weight of 100,000 to 1,000,000) and F₃ - (having a molecular weight of 10,000 to 100,000) prepared from RON obtained in Example 4 by partial hydrolysis using 2% sulfuric acid given hereinafter was administered are described in detail.

(1) Antitumor Activities

(a) Effect of Intraperitoneal Administration of the RON Substance against Syngeneic Tumor Meth-A

To the peritoneal cavities of 6-week-old female BALB/C-CRJ mice having an average body weight of 20 g, Meth-A tumor cells subcultured in the peritoneal cavities of the same syngeneic mice were transplanted at a rate of 1×10^5 cells per mouse. The mice were divided into one control group consisting of 20 mice and three test groups, each consisting of 10 mice. Continuously for 5 days from the day subsequent to the transplanta-
55 tion of the tumor cells, 0.1 ml each of physiological saline solutions containing 10 mg, 30 mg and 100 mg of RON per kg of the body weight of one mouse were administered intraperitoneal by cavities to the mice of test groups, respectively, and only a physiological saline solution alone was similarly administered to the mice of the control group. Then, the survival days were observed, and the prolongation

of life was calculated according to the following formula:

$$\text{Prolongation of life (\%)} = \frac{\text{average survival time (days) for test group}}{\text{average survival time (days) for control group}} \times 100$$

(b) Effect of Oral Administration of the RON Substance against Syngeneic Tumor Meth-A

In the axillary regions of 6-week-old female BALB/C-CRJ mice having an average body weight of 20 g, Meth-A tumor cells were subcutaneously transplanted at a rate of 6×10^4 cells per mouse. The mice were divided into one control group consisting of 20 mice and three test groups, each consisting of 10 mice. Continuously for 10 days from the day subsequent to transplantation of the tumor cells, 0.2 ml each of physiological saline solutions containing 10, 30 and 100 mg of the RON substance per kg of the body weight of one mouse were administered orally by using an oral sonde. Only a physiological saline was similarly administered to the mice of the control group. After 35 days from the transplantation of the tumor cells, each mouse was killed, and the propagated tumor was cut out and the weight was measured. The inhibition ratio was calculated according to the following formula:

$$\text{Inhibition ratio (\%)} = \left(1 - \frac{\text{average tumor weight in test group}}{\text{average tumor weight in control group}} \right) \times 100$$

The antitumor activities of the RON substance tested according to the above-mentioned methods (a) and (b) are shown in Table I.

Table I

| Sample | Dose (mg/kg) | Intraperitoneal Administration (a) | | Oral Administration(b) | |
|------------------|--------------|------------------------------------|--------------------------|--------------------------|----------------------|
| | | average survival time (days) | Prolongation of life (%) | average tumor weight (g) | inhibition ratio (%) |
| control (saline) | - | 19.8 | - | 11.00 | - |
| RON substance | 10 | 32.1 | 162 | 5.98 | 46 |
| RON substance | 30 | >42.0 | >212 | 4.26 | 61 |
| RON substance | 100 | 31.8 | 161 | 5.05 | 54 |

From the results shown in the Table I, it can be seen that the RON substance exhibits a strong antitumor activity with the optimum dose of about 30 mg/kg both in the intraperitoneal and in the oral administration.

The same procedure as above described (a) and (b) was carried out using the low-molecular-weight products of RON substance F_1 , F_2 and F_3 in stead of using the RON. The results are shown in Table II.

Table II

| Sample | Dose (mg/kg) | Intraperitoneal Administration (a) | | Oral Administration (b) | |
|------------------|--------------|------------------------------------|--------------------------|--------------------------|----------------------|
| | | average survival time (days) | prolongation of life (%) | average tumor weight (g) | inhibition ratio (%) |
| Control (saline) | | 19.8 | - | 11.00 | - |
| F ₁ | 30 | >42.0 | >212 | 4.39 | 60 |
| F ₂ | 30 | >42.0 | >212 | 4.28 | 61 |
| F ₃ | 30 | >42.0 | >212 | 4.79 | 56 |

From the result shown in Table II, it is found that the low-molecular-weight products of the RON substance have an antitumor activity which is equal with the RON substance before partial hydrolysis.

Furthermore, it was confirmed that when the RON substance and low-molecular-weight products thereof were intraperitoneally or orally administered at a dose of 10 to 100 mg/kg, activities corresponding to a tumor inhibition ratio of 30 to 70% is attained for the murine syngeneic tumors Lewis lung carcinoma, Melanoma B-16, tumors Sarcoma-180 and Ehrlich ascites tumor. Moreover, it was confirmed that when the RON substance and low-molecular-weight product thereof were administered to mice in combination with an appropriate primer, the cytotoxicity to L-929 cells and the necrotic activity to Meth-A solid tumor were induced in the serum, and a tumor necrosis factor was self-induced in the living body of a tumor-bearing mice. In view of the fact that the RON substance and low-molecular-weight products thereof have no toxicity at all as described hereinafter, it is considered that the RON substance can be a very effective antitumor agent.

(2) Immunomodulating Activities

(a) Carbon Clearance Test (CCT)

This test is used to examine the effect of enhancing the phagocytic activity of the macrophage. A physiological saline solution containing the RON substance or low-molecular weight products thereof such as F₁, F₂ and F₃ dissolved therein was intraperitoneally administered for 2 days into one group of six 4-week-old female ICR-CRJ mice having an average body weight of 20 g (only a physiological saline was administered to the control group), and on the third day, 0.25 ml of a carbon solution [prepared by diluting a black ink (Fount India® supplied by Pelikan Co.) 5 times with a physiological saline solution] was injected into the tail vein of the mice. Just after the injection and after 10 minutes, 0.025 ml of blood was collected from the venous plexus of the retro-orbit of the mice and suspended and dissolved in 3.5 ml of a 0.01 M solution of sodium carbonate. The absorbance (OD₆₅₀) at 650 nm was measured and the reduction ratio of the carbon concentration in blood was examined. The effect was expressed by the phagocytic index represented by the following formula:

$$\text{Phagocytic index (K)} = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

wherein C₁ represents OD₆₅₀ at T₁ and C₂ represents OD₆₅₀ at T₂.

In case of tumor-bearing mice, 7 days before initiation of administration of the RON substance, F₁, F₂ or F₃ 1 x 10⁷ of Sarcoma-180 cells were transplanted in the muscle of the hind leg of each mouse, and the test was similarly carried out. The obtained results are shown in Table III. It was found that in case of both normal mice and tumor-bearing mice, the function of the reticuloendothelial system of the mice was enhanced by administration of 10 to 100 mg/kg, especially 30 mg/kg, of the RON substance, F₁, F₂ or F₃. It is clear that the phagocytic activity of the macrophage has been increased.

Table III

| Sample | Dose (mg/kg) | Normal Mice | | Tumor-Bearing Mice | |
|------------------|--------------|-----------------------|-------------------|----------------------|-------------------|
| | | phagocytic index | relative value(%) | phagocytic index | relative value(%) |
| control (saline) | - | 40.2x10 ⁻³ | 100 | 104x10 ⁻³ | 100 |
| RON substance | 10 | 58.3x10 ⁻³ | 145 | 129x10 ⁻³ | 124 |
| RON substance | 30 | 90.0x10 ⁻³ | 224 | 159x10 ⁻³ | 153 |
| Ron substance | 100 | 77.6x10 ⁻³ | 193 | 146x10 ⁻³ | 140 |
| F ₁ | 30 | 90.8x10 ⁻³ | 226 | 159x10 ⁻³ | 153 |
| F ₂ | 30 | 87.2x10 ⁻³ | 217 | 157x10 ⁻³ | 151 |
| F ₃ | 30 | 89.9x10 ⁻³ | 224 | 160x10 ⁻³ | 154 |

(b) Plaque-Forming Cell Test (PFC)

This test is used to examine the effect of enhancing the antibody-producing ability by activation of B cells of the host among the immunomodulating activities.

A physiological saline solution containing the RON substance or low-molecular-weight products thereof such as F₁, F₂ and F₃ was continuously administered for 3 days intraperitoneally cavities to one group of six 4-week-old female ICR-CRJ mice having an average body weight of 20 g (only a physiological saline was administered to the control group). On the 4th day and 11th day, 4 x 10⁶ of sheep erythrocytes were injected into the tail vein, and after 4 days, the plaque-forming ability of the mouse spleen cells was determined according to the Cunningham method.

The obtained results are shown in Table IV. It is seen that the antibody-producing ability in highly enhanced by the administration of 10 to 100 mg/kg of the RON substance, F₁, F₂ or F₃.

Table IV

| Sample | Dose (mg/kg) | Sensitization on 4th Day | | Sensitization on 11th Day | |
|------------------|--------------|------------------------------|--------------------|------------------------------|--------------------|
| | | number of plaques per spleen | relative value (%) | number of plaques per spleen | relative value (%) |
| control (saline) | - | 3.0x10 ⁴ | 100 | 2.4x10 ⁴ | 100 |
| RON substance | 10 | 4.6x10 ⁴ | 153 | 6.5x10 ⁴ | 271 |
| RON substance | 30 | 7.6x10 ⁴ | 253 | 8.9x10 ⁴ | 311 |
| RON substance | 100 | 7.1x10 ⁴ | 237 | 8.0x10 ⁴ | 333 |
| F ₁ | 30 | 7.7x10 ⁴ | 257 | 8.8x10 ⁴ | 367 |
| F ₂ | 30 | 7.4x10 ⁴ | 247 | 8.4x10 ⁴ | 350 |
| F ₃ | 30 | 7.1x10 ⁴ | 237 | 8.1x10 ⁴ | 338 |

(c) Delayed Tyep Hypersensitivity Reaction (DHR)

This test is used to examine the effect of enhancing the cell-mediated immunity action by the activation of T cells of the host among the immunomodulating activities.

A physiological saline containing the RON substance or low-molecular-weight products such as F₁, F₂ or F₃ was orally administered to one group of six 8-week-old female ICR-CRJ mice having an average body weight of 27 g (only a physiological saline was administered to the control group). On the 4th day after the initiation of administration, a 5% ethanol solution of picryl chloride was coated on the abdominal region which had been shaved to effect the primary sensitization. On the 11th day, a 1% olive oil solution of picryl chloride was coated on the front and back sides of both the ears of each mouse to effect the secondary sensitization. After 24 hours, the ear thickness was measured by a gauge, and the increase of the ear thickness was determined from the difference between the ear thickness before and after the coating. In case of tumor-bearing mice, 1 x 10⁵ of Sarcoma 180 ascitic tumor cells were transplanted into the

peritoneal cavity of the mouse one day before the administration. Then, the test was similarly carried out.

The obtained results are shown in Table V. It is seen that in case of both normal mice and tumor-bearing mice, the cell-mediated immunity action is highly enhanced by the oral administration of 30 to 500 mg/kg of the RON substance, F₁, F₂ or F₃.

Table V

| Sample | Dose (mg/kg) | Normal Mice | | Tumor-Bearing Mice | |
|------------------|--------------|--------------------------------|--------------------|--------------------------------|--------------------|
| | | increase of ear thickness (μm) | relative value (%) | increase of ear thickness (μm) | relative value (%) |
| control (saline) | - | 32.1 | 100 | 39.7 | 100 |
| RON substance | 30 | 75.2 | 234 | 26.4 | 272 |
| RON substance | 100 | 70.2 | 219 | 24.3 | 251 |
| RON substance | 500 | 61.3 | 191 | 20.5 | 211 |
| F ₁ | 30 | 75.3 | 235 | 25.9 | 267 |
| F ₂ | 30 | 71.8 | 224 | 25.1 | 259 |
| F ₃ | 30 | 70.0 | 218 | 25.0 | 258 |

From the results of the immunity action tests (a), (b) and (c), it is seen that various immunity actions in mice having different mechanisms can be conspicuously enhanced by the RON substance and low-molecular-weight products such as F₁, F₂ and F₃. Since an immunomodulating agent is generally used when the immunological competence of a living body is reduced or the foreign antigen-recognizing function is poor, it is expected that the RON substance and low-molecular-weight products such as F₁, F₂ and F₃ will be effectively used as a therapeutic agent, adjuvant therapeutic agent, combined therapeutic agent, preventing agent or recuperation-accelerating agent after the operation against bacterial and virus infectious diseases and malignant tumors. In addition to the above-mentioned immunity activating and recovering actions, the immunomodulating agent exerts a function of normalizing an abnormally enhanced immunoreaction in a living body, and it is considered that the RON substance and low-molecular-weight products such as F₁, F₂ and F₃ will be applied to self-immunity diseases such as rheumatism, collagen diseases and allergic diseases:

(3) Host Defense Activity

In general, a living body has a sufficient defense activity against the invasion of foreign bacteria. It is known that in the cancer-bearing state, especially at the later stage of cancer, the defense activity is drastically reduced and a serious damage is incurred even by non-pathogenic bacteria ordinarily symbiotic with the host.

Accordingly, in order to examine whether or not the RON substance and low-molecular-weight products such as F₁, F₂ and F₃ can enhance the defense activity of the host against these bacterial infectious diseases, the effect of the RON substance, F₁, F₂ and F₃ on the infection with *Escherichia coli* and *Listeria monocytogenes* was examined.

A physiological saline solution containing 10 to 100 mg/kg of the RON substance, F₁, F₂ or F₃ was administered subcutaneously to the backs of one group of twenty 7-week-old female ICR-CRJ mice having an average body weight of 26 g (only physiological saline was administered to the control group) once one day before the infection with the bacteria and once one day after the infection with the bacteria. In case of *Escherichia coli*, 2 x 10⁷ of cells were transplanted subcutaneously on the back, and in case of *Listeria monocytogenes*, 2 x 10⁷ of cells were transplanted intraperitoneally. Then, the mice were observed for 1 week, and the number of survived mice was counted and the protective effect was calculated according to the following formula:

$$\text{Protective effect (\%)} = \frac{(\text{number of survival mice in test group}) - (\text{number of survival mice in control group})}{\text{number of mice in one group}} \times 100$$

The obtained results are shown in Table VI. It is seen that when 10 to 100 mg/kg of the RON substance, F₁, F₂ or F₃ is administered before the infection with Escherichia coli, a very strong defense activity against the infection with Escherichia coli can be attained, and a significant effect of enhancing the defense activity against the infection with Listeria monocytogenes. In case of the administration after the
5 infection, the RON substance has a significant therapeutic effect against both the infectious bacteria.

In view of the fact that the RON substance, F₁, F₂ and F₃ have no toxicity as illustrated hereinafter, it is considered that the RON substance and low-molecular-weight products such as F₁, F₂ and F₃ can be very useful host defense agent against infectious diseases.

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Table VI

| Infectious Bacterium | Sample | Dose (mg/kg) | One Day before Infection | | One Day after Infection | |
|--|------------------|-----------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|
| | | | number of survival mice | protective effect (%) | number of survival mice | protective effect (%) |
| <u>Escherichia coli</u> * | control (saline) | - | 0 | - | 0 | - |
| ditto | RON substance | 10 | 16 | 80 | 11 | 55 |
| ditto | RON substance | 30 | 18 | 90 | 14 | 70 |
| ditto | RON substance | 100 | 18 | 90 | 14 | 70 |
| ditto | F ₁ | 30 | 18 | 90 | 14 | 70 |
| ditto | F ₂ | 30 | 17 | 85 | 13 | 65 |
| ditto | F ₃ | 30 | 17 | 85 | 11 | 55 |
| <u>Listeria mono- cytogenes</u> ** | control (saline) | - | 0 | - | 0 | - |
| ditto | RON substance | 10 | 7 | 35 | 8 | 40 |
| ditto | RON substance | 30 | 15 | 75 | 13 | 65 |
| ditto | RON substance | 100 | 13 | 65 | 12 | 60 |
| ditto | F ₁ | 30 | 14 | 70 | 13 | 65 |
| ditto | F ₂ | 30 | 14 | 70 | 14 | 70 |
| ditto | F ₃ | 30 | 12 | 60 | 12 | 60 |

Note

* : Escherichia coli SB-001** : Listeria monocytogenes SB-010

The acute toxicity of the RON substance will now be described. The RON substance, F₁, F₂ and F₃ were orally administered to one group of ten 5-week-old male SD-CRJ rats having a body weight of 120 to 150 g at a dose of 15 g/kg, which is a physical administration limit dose, and the rats were observed. None of the rats died, and the increase of the body weight was not different from that in the control group and no abnormality was observed in both appearance and necropsy. Accordingly, it is judged that LD₅₀ is larger than 15 g/kg and the RON substance, F₁, F₂ and F₃ have no acute toxicity.

When the RON substance was intravenously injected, LD₅₀ was 300 mg/kg, and in case of F₁, F₂ and F₃, the toxicity decreased with decrease of the molecular weight. In case of F₃, LD₅₀ was larger than 5 g/kg and no toxicity was observed. Accordingly, the molecular weight-reduced RON substance is very advantageous when used as an injection drug.

5 According to the present invention, the RON substance having the above-mentioned excellent antitumor activity, immunomodulating activity host defense activity against infections can be obtained stably in a large quantity as a fermentation product by a microorganism, and a pure product can be obtained by relatively easy methods. Accordingly, the present invention makes a great contribution to the technique of producing the present RON substance by using a microorganism on an industrial scale.

10 Furthermore, the RON substance and low-molecular-weight products thereof have functions of enhancing the cytotoxicity of natural killer cells derived from spleen cells of rats and activating the cytotoxic activity against L-929 cells of resident peritoneal macrophages of a mouse. Moreover, the RON substance has an immunity activating ability and a function of enhancing a production of a cytokine such as interferon, and therefore, it is expected that the RON substance will exert preventive and therapeutic effects against virus
15 diseases such as herpes, influenza and AIDS. Still further, it is considered that the RON substance will be useful as a preventive and therapeutic agent against hepatitis such as chronic hepatitis and liver diseases.

Since the RON substance, F₁, F₂ and F₃ can be orally or non-orally administered, it is expected that the RON substance will be effectively used as an antitumor agent, immunomodulating agent or preventive or therapeutic agent against infectious diseases.

20 In the practical production of medicines, the RON substance, F₁, F₂ or F₃ is used alone or combined with an excipient (such as water, physiological saline, polyethylene glycol, glycerol, starch, dextrin or lactose) and formed into a liquid preparation, a pill, a tablet, a powder or a suppository.

Since the RON substance has no toxicity, shows various biological activities valuable for the maintenance of health by oral administration, has no taste or smell and can be easily processed, therefore the
25 RON substance can be used as a food or drink for prevention of diseases or maintenance of the health or as an additive to foods or drinks in addition to medicines as mentioned above.

According to the present invention, the RON substance can be stably obtained in a high yield as a fermentation product by a microorganism. There are few of substances having no toxicity and having excellent antitumor activity, immunomodulating activity and host defense activity against infections, like the
30 RON substance obtained according to the present invention. Therefore, the present invention is industrially very advantageous and valuable.

[Examples]

35 The present invention will now be described in detail with reference to the following examples that by no means limit the scope of the invention.

Example 1

40 (Culturing)

Cells obtained by the stab culture of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242) were inoculated into 8 ml of a culture medium (comprising 2.0% of sucrose, 0.5% of yeast extract and 2.0% of K₂HPO₄ and having a pH value of 7.4) charged in a test tube having a diameter of 15
45 mm. The stationary culture was carried out at 26 °C for 24 hours. Then, 8 ml of the culture broth was charged in an Erlenmeyer flask having a volume of 500 ml, in which 400 ml of the culture medium having the same composition as described above was charged, and the stationary culture was carried out at 26 °C for 24 hours. Then, 400 ml of the culture broth was inoculated in 20 l of SM culture medium (comprising 15% of sucrose, 0.05% of yeast extract, 0.5% of K₂HPO₄ and 0.1% of NaCl and having a pH value of 7.4),
50 which was charged in a jar fermentator having a volume of 30 l, and cultured at 30 °C for 15 hours under circulation of nitrogen with gentle stirring (0.2 v/v/min, agitation of 10 rpm).

(Purification)

55 After the pH value of the obtained culture broth was adjusted to 7, 20 l of the culture broth was heated at 100 °C for the sterilization, and the cells and insoluble substances were removed by a continuous centrifugal separator to obtain 18 l of a culture supernatant.

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Methanol was gradually added to the obtained culture supernatant so that the final concentration was 40% (v/v), and the mixture was allowed to stand still. The supernatant was removed by decantation and the obtained precipitate was washed with 60% (v/v) methanol. The precipitate was dissolved in 18 l of deionized water, and precipitation with methanol and washing with 60% (v/v) methanol were carried out to
5 obtain 2.8 kg of a glutinous white precipitate. The precipitate was dissolved in 15 l of deionized water, and the solution was spray-dried to obtain 550 g of a white powder of the RON substance.

Example 2

10 Culture and removal of cells by the centrifugal separation and methanol precipitation were carried out in the same manner as described in Example 1 except that 10% of blackstrap molasses was added instead of sucrose to the SM culture medium used in Example 1, whereby about 2.5 kg of a glutinous precipitate was obtained. The obtained precipitate was dissolved in a small amount of deionized water, and the solution was
15 dropped into acetone. The obtained precipitate was vacuum-dried to obtain 490 g of a white powder of the RON substance.

Example 3

Methanol was gradually added to 100 ml of the culture supernatant obtained in Example 1 so that the
20 final concentration was 40% (v/v), and the obtained glutinous precipitate was washed with 60% (v/v) methanol and dissolved in 100 ml of deionized water. The solution was applied to DEAE-Toyopearl 650M® equilibrated with water, and passed fractions were collected and subjected to ultrafiltration with a membrane having an exclusion molecular weight of 100,000, followed by concentration and desalting, and freeze drying, to obtain 2.2 g of a white powder of the RON substance.

Example 4

In 100 ml of 2% sulfuric acid was dissolved 2.0 g of the white powder of the RON substance obtained in Example 1, and partial hydrolysis was carried out at 60°C for 4 hours. The reaction mixture was
30 neutralized with BaCO₃, and the precipitate was removed by centrifugal separation. Ultrafiltration was carried out in sequence by membranes having exclusion molecular weights of 1,000,000, 100,000 and 10,000, respectively, to obtain three fractions, that is, fraction F₁ (having a molecular weight higher than 1,000,000), fraction F₂ (having a molecular weight of 100,000 to 1,000,000) and fraction F₃ (having a molecular weight of 10,000 to 100,000). These fractions were freeze-dried to obtain white powder. The yield
35 was about 450 mg of F₁, about 700 mg of F₂ and about 500 mg of F₃, respectively.

Example 5

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, 46-1 (FERM BP-2670) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 520 g of the RON substance in the form of a white powder was obtained.

Example 6

45 The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, NCFB 517 (FERM BP-2711) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 99 g of the RON substance in the form of a white powder was obtained.

Example 7

50 The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, NCFB 531 (FERM BP-2712) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 6 g of the RON substance in the form of a white powder was obtained.

Example 8

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, NCFB 861 (FERM BP-2713) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 3 g of the RON substance in the form of a white powder was obtained.

Example 9

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, NCFB 864 (FERM BP-2714) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 85 g of the RON substance in the form of a white powder was obtained.

Example 10

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, NCFB 880 (FERM BP-2715) was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 123 g of the RON substance in the form of a white powder was obtained.

Example 11

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, ATCC 1956 was used instead of Leuconostoc mesenteroides subsp. dextranicum BL-75 (FERM BP-2242), whereby 171 g of the RON substance in the form of a white powder was obtained.

Example 12

The procedures of Example 1 were repeated in the same manner except that Leuconostoc mesenteroides subsp. dextranicum, IFO 3349 was used instead of Leuconostoc mesenteroides subsp. dextranicum, BL-75 (FERM BP-2242), whereby 108 g of the RON substance in the form of a white powder was obtained.

Example 13

(Culturing)

Cells obtained by the stab culture of Leuconostoc mesenteroides subsp. dextranicum, BL-75 were inoculated into 8 ml of a culture medium (comprising 2.0% of sucrose, 0.5% of yeast extract and 2.0% of K_2HPO_4 and having a pH value of 7.4) charged in a test tube having a diameter of 15 mm. The stationary culture was carried out at 26 °C for 24 hours. Then, 8 ml of the culture broth was charged in an Erlenmeyer flask having a volume of 500 ml, in which 400 ml of the culture medium having the same composition as described above was charged, and the stationary culture was carried out at 26 °C for 24 hours. Then, 40 g of sucrose were added to 400 ml of the culture liquid and the mixture was stirred to dissolve, and the culture was conducted at 30 °C for 24 hours.

(Purification)

After the pH value of the obtained culture broth was adjusted to 7, 400 ml of the culture broth was heated at 100 °C for the sterilization, after dilution of the culture broth for two times with water and the cells and insoluble substances were removed by a centrifugal separator to obtain 700 ml of a culture supernatant.

Methanol was gradually added to the obtained culture supernatant so that the final concentration was 40% (v/v), and the mixture was allowed to stand still. The supernatant was removed by decantation and the obtained precipitate was washed with 60% (v/v) methanol. The precipitate was dissolved in 18 l of deionized water, and precipitation with methanol and washing with 60% (v/v) methanol were carried out to

obtain 60 g of a glutinous white precipitate. The precipitate was dissolved in 400 ml of deionized water, and the solution was spray-dried to obtain 10 g of a white powder of the RON substance.

Example 14

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Cells in the culture broth (400 ml) obtained in the same manner as described in Example 13 were pulverized by an ultrasonic pulverizer and insoluble substances were removed by a centrifugal separator to obtain 350 ml of a processed culture broth. Then, 40 g of sucrose was added to the obtained processed culture broth, and the mixture was stirred to dissolve sucrose. The solution was allowed to stand at 30 °C for 24 hours and diluted 2 times with water. Methanol was gradually added to the dilution with stirring so that the final concentration was 40% (v/v), and the mixture was allowed to stand for a while. The supernatant was removed by decantation, and the obtained precipitate was washed with 60% (v/v) methanol. The precipitate was dissolved in 700 ml of deionized water, and precipitation with methanol and washing with 60% (v/v) methanol were repeated, whereby a glutinous precipitate was obtained. The precipitate was dissolved in 400 ml of deionized water, and the solution was freeze-dried to obtain 12 g of a white powder of the RON substance.

Example 15

20 The procedures of Example 14 were repeated in the same manner except that the culture broth was not pulverized by the ultrasonic pulverizer, whereby 13 g of a white powder of the RON substance was obtained.

Example 16

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The processed culture broth obtained by subjecting the culture broth to centrifugal separation in Example 15 was dialyzed at 4 °C overnight against 50 mM phosphate buffer having a pH value of 5.5. Other operations were same as those conducted in Example 15. As the result, 13.5 g of a white powder of the RON substance was obtained.

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Claims

Claims for the following Contracting States : CH, DE, FR, GB, IT, LI

1. Use of a strain selected from the group consisting of *Leuconostoc mesenteroides* subsp. *dextranicum*, strains BL-75 (FERM BP-2242), strain NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 and IFO 3349, and mutants thereof, for producing in a culture medium containing sucrose as a source of carbon physiologically active polysaccharide RON substance having the following properties:
 - (1) Form: the substance is a white amorphous powder having no taste and no smell;
 - (2) Solubility: the substance is soluble in water, but the solution becomes milky white and viscous when the concentration is elevated, the substance is soluble in formamide and dimethylsulfoxide, but the substance is insoluble in alcohols, acetone, benzene, ethyl acetate, hexane, chloroform and carbon tetrachloride;
 - (3) pH of Aqueous Solution: the aqueous solution is neutral or weakly acidic;
 - (4) Constituent Saccharide: the substance is composed solely of glucose;
 - (5) Elementary Analysis Values: the substance comprises 44.0 to 45.0 % of C and 6.1 to 6.3 % of H;
 - (6) Structure: the substance is an α -1,6-glucan and contains a small amount of 3,6-branched side chains;
 - (7) Proteins: the substance contains almost no proteins;
 - (8) Molecular Weight: the substance does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
 - (9) Specific Rotation: $[\alpha]_D^{25} = +190^\circ \sim +220^\circ$ (C=0.5, formamide);
 - (10) Color Reaction: the substance is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
 - (11) Melting Point: the substance has no definite melting point;
 - (12) Ultraviolet Absorption Spectrum: the substance has no characteristic absorption;

- (13) Infrared Absorption Spectrum; the substance shows a characteristic absorption of α -glucan;
- (14) ^{13}C -NMR Spectrum: the substance shows absorption characteristic chemical shift values of α -1,6-glucan in the main signals; and
- (15) Biological Activity: the substance has an antitumor activity.

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2. A biologically pure culture of the microorganisms *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) and *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670).

Claims for the following Contracting State : ES

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1. A process for the production of biologically active polysaccharide RON substance which comprises culturing a microorganism strain selected from the group consisting of *Leuconostoc mesenteroides* subsp. *dextranicum*, strains BL-75 (FERM BP-2242), strain NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 and IFO 3349, and mutants thereof,

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for producing in a culture medium containing sucrose as a source of carbon physiologically active polysaccharide RON substance having the following properties, and collecting the physiologically active polysaccharide RON substance from the culture broth:

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- (1) Form: the substance is a white amorphous powder having no taste and no smell;
- (2) Solubility: the substance is soluble in water, but the solution becomes milky white and viscous when the concentration is elevated, the substance is soluble in formamide and dimethylsulfoxide, but the substance is insoluble in alcohols, acetone, benzene, ethyl acetate, hexane, chloroform and carbon tetrachloride;

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- (3) pH of Aqueous Solution: the aqueous solution is neutral or weakly acidic;
- (4) Constituent Saccharide: the substance is composed solely of glucose;
- (5) Elementary Analysis Values: the substance comprises 44.0 to 45.0 % of C and 6.1 to 6.3 % of H;
- (6) Structure: the substance is an α -1,6-glucan and contains a small amount of 3,6-branched side chains;

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- (7) Proteins: the substance contains almost no proteins;
- (8) Molecular Weight: the substance does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;

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- (9) Specific Rotation: $[\alpha]_D^{25} = +190^\circ \sim +220^\circ$ (C=0.5, formamide);
- (10) Color Reaction: the substance is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;

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- (11) Melting Point: the substance has no definite melting point;
- (12) Ultraviolet Absorption Spectrum: the substance has no characteristic absorption;
- (13) Infrared Absorption Spectrum: the substance shows a characteristic absorption of α -glucan;
- (14) ^{13}C -NMR Spectrum: the substance shows absorption characteristic chemical shift values of α -1,6-glucan in the main signals; and
- (15) Biological Activity: the substance has an antitumor activity.

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2. The process according to claim 1, wherein as the microorganism strain *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) or *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670) is used.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : CH, DE, FR, GB, IT, LI

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1. Verwendung eines Stammes, ausgewählt aus der Gruppe, die besteht aus *Leuconostoc mesenteroides* subsp. *dextranicum*, Stamm BL-75 (FERM BP-2242), Stamm NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 und IFO 3349 und Mutanten davon,

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zur Herstellung von physiologisch aktiver Polysaccharid-RON-Substanz mit den nachstehend angegebenen Eigenschaften in einem Kulturmedium, das Saccharose als Kohlenstoffquelle enthält:

- (1) Form: die Substanz ist ein weißes amorphes Pulver ohne Geschmack und ohne Geruch;

- (2) Löslichkeit: die Substanz ist in Wasser löslich, die Lösung wird jedoch milchig-weiß und viskos, wenn die Konzentration erhöht wird, die Substanz ist in Formamid und Dimethylsulfoxid löslich, die Substanz ist jedoch in Alkoholen, Aceton, Benzol, Ethylacetat, Hexan, Chloroform und Tetrachlorkohlenstoff unlöslich;
- 5 (3) pH-Wert der wässrigen Lösung: die wässrige Lösung ist neutral oder schwach sauer;
- (4) Saccharid-Bestandteil: die Substanz besteht nur aus Glucose;
- (5) Werte der Elementar-Analyse: die Substanz enthält 44,0 bis 45,0 % C und 6,1 bis 6,3 % H;
- (6) Struktur: die Substanz ist ein α -1,6-Glucan und enthält eine geringe Menge 3,6-verzweigte Seitenketten;
- 10 (7) Proteine: die Substanz enthält nahezu keine Proteine;
- (8) Molekulargewicht: die Substanz durchdringt nicht eine Dialysemembran und ihr Molekulargewicht ist, wie angenommen wird, höher als 10 000 Dalton;
- (9) spezifische Drehung: $[\alpha]_D^{25} = +190^\circ$ bis $+220^\circ$ (C=0,5, Formamid);
- 15 (10) Farbreaktion: die Substanz ist positiv gegenüber der Anthron-Schwefelsäure-Reaktion und der Phenol-Schwefelsäure-Reaktion, jedoch negativ gegenüber der Biuret-Reaktion, der Lowry-Folin-Reaktion, der Elson-Morgan-Reaktion und der Jod-Reaktion;
- (11) Schmelzpunkt: die Substanz hat keinen definierten Schmelzpunkt;
- (12) Ultraviolett-Absorptionsspektrum: die Substanz weist keine charakteristische Absorption auf;
- (13) Infrarot-Absorptionsspektrum: die Substanz zeigt die charakteristische Absorption von α -Glucan;
- 20 (14) ^{13}C -NMR Spektrum: die Substanz weist in den Hauptsignalen die Absorptions-charakteristischen chemischen Verschiebungs-Werte von α -1,6-Glucan auf; und
- (15) biologische Aktivität: die Substanz weist eine Antitumor-Aktivität auf.
2. Biologisch reine Kultur der Mikroorganismen *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) und *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670).
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Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung von biologisch aktiver Polysaccharid-RON-Substanz, das umfaßt das Kultivieren eines Mikroorganismen-Stammes, ausgewählt aus der Gruppe, die besteht aus
- 30 *Leuconostoc mesenteroides* subsp. *dextranicum*, Stamm BL-75 (FERM BP-2242), Stamm NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670), ATCC 1956 und IFO 3349 und Mutanten davon,
- 35 zur Bildung von physiologisch aktiver Polysaccharid-RON-Substanz, welche die nachstehend angegebenen Eigenschaften hat, in einem Kulturmedium, das Saccharose als Kohlenstoffquelle enthält, und das Sammeln der physiologisch aktiven Polysaccharid-RON-Substanz aus der Kulturbrühe:
- (1) Form: die Substanz ist ein weißes amorphes Pulver ohne Geschmack und ohne Geruch;
- 40 (2) Löslichkeit: die Substanz ist in Wasser löslich, die Lösung wird jedoch milchig-weiß und viskos, wenn die Konzentration erhöht wird, die Substanz ist in Formamid und Dimethylsulfoxid löslich, die Substanz ist jedoch in Alkoholen, Aceton, Benzol, Ethylacetat, Hexan, Chloroform und Tetrachlorkohlenstoff unlöslich;
- (3) pH-Wert der wässrigen Lösung: die wässrige Lösung ist neutral oder schwach sauer;
- (4) Saccharid-Bestandteil: die Substanz besteht nur aus Glucose;
- 45 (5) Werte der Elementar-Analyse: die Substanz enthält 44,0 bis 45,0 % C und 6,1 bis 6,3 % H;
- (6) Struktur: die Substanz ist ein α -1,6-Glucan und enthält eine geringe Menge 3,6-verzweigte Seitenketten;
- (7) Proteine: die Substanz enthält nahezu keine Proteine;
- (8) Molekulargewicht: die Substanz durchdringt nicht eine Dialysemembran und ihr Molekulargewicht ist, wie angenommen wird, höher als 10 000 Dalton;
- 50 (9) spezifische Drehung: $[\alpha]_D^{25} = +190^\circ$ bis $+220^\circ$ (C=0,5, Formamid);
- (10) Farbreaktion: die Substanz ist positiv gegenüber der Anthron-Schwefelsäure-Reaktion und der Phenol-Schwefelsäure-Reaktion, jedoch negativ gegenüber der Biuret-Reaktion, der Lowry-Folin-Reaktion, der Elson-Morgan-Reaktion und der Jod-Reaktion;
- 55 (11) Schmelzpunkt: die Substanz hat keinen definierten Schmelzpunkt;
- (12) Ultraviolett-Absorptionsspektrum: die Substanz weist keine charakteristische Absorption auf;
- (13) Infrarot-Absorptionsspektrum: die Substanz zeigt die charakteristische Absorption von α -Glucan;

(14) ^{13}C -NMR Spektrum: die Substanz weist in den Hauptsignalen die Absorptions-charakteristischen chemischen Verschiebungs-Werte von α -1,6-Glucan auf; und

(15) biologische Aktivität: die Substanz weist eine Antitumor-Aktivität auf.

- 5 2. Verfahren nach Anspruch 1, worin als Mikroorganismenstamm *Leuconostoc mesenteroides* subsp. *dextranicum* BL-75 (FERM BP-2242) oder *Leuconostoc mesenteroides* subsp. *dextranicum* 46-1 (FERM BP-2670) verwendet wird.

Revendications

10 Revendications pour les Etats contractants suivants : CH, DE, FR, GB, IT, LI

1. Utilisation d'une souche choisie au sein du groupe constitué par les souches de *Leuconostoc mesenteroides*, sous-espèce *dextranicum*, BL-75 (FERM BP-2242), NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670) ATCC 1956 et IFO 3349 ainsi que leurs formes mutantes,
 - 15 pour produire dans un milieu de culture contenant du saccharose comme source de carbone de la substance polysaccharidique RON biologiquement active possédant les propriétés suivantes :
 - (1) Forme : la substance est une poudre amorphe de couleur blanche, inodore et sans saveur;
 - (2) Solubilité : la substance est hydrosoluble, mais elle prend un aspect blanc laiteux et visqueux lorsque la concentration est élevée; la substance est soluble dans le formamide et dans le diméthylsulfoxyde, mais elle est insoluble dans l'alcool, l'acétone, le benzène, l'acétate d'éthyle, l'hexane, le chloroforme et le tétrachlorure de carbone;
 - (3) pH de la solution aqueuse : la solution aqueuse est neutre ou légèrement acide;
 - (4) Constituant saccharidique : la substance est uniquement composée de glucose;
 - 25 (5) Analyse élémentaire: la substance comprend 44,0% à 45,0% de C et 6,1 à 6,3% de H;
 - (6) Structure: la substance est un α -1,6-glycane qui contient une petite quantité de chaînes latérales ramifiées en 3,6;
 - (7) Protéines : la substance ne contient pratiquement pas de protéines;
 - (8) Poids moléculaire : la substance ne traverse pas une membrane de dialyse, son poids moléculaire est donc supposé être supérieur à 10.000 daltons;
 - 30 (9) Rotation spécifique : $[\alpha]_D^{25} = +190^\circ \sim +220^\circ$ (C = 0,5, formamide);
 - (10) Réaction colorimétrique : la substance donne un résultat positif à la réaction avec l'anthrone-acide sulfurique et à la réaction avec le phénol-acide sulfurique, mais un résultat négatif à la réaction du biuret, à la réaction de Lowry-Folin, à la réaction d'Elson-Morgan et à la réaction avec l'iode;
 - 35 (11) Point de fusion: la substance n'a pas de point de fusion défini;
 - (12) Spectre d'absorption dans l'ultraviolet : la substance n'a pas d'absorption caractéristique;
 - (13) Spectre d'absorption dans la l'infrarouge : la substance présente une absorption caractéristique de l' α -glycane;
 - 40 (14) Spectre de RMN du ^{13}C : la substance montre les valeurs de transition chimique caractéristiques de l' α -1,6-glycane pour les principaux signaux et
 - (15) Activité biologique : la substance a une activité anti-tumorale.
2. Culture biologiquement pure de micro-organismes de la souche de *Leuconostoc mesenteroides*, sous-espèce *dextranicum* BL-75 (FERM BP-2242) et de la souche de *Leuconostoc mesenteroides*, sous-espèce *dextranicum*, 46-1 (FERM BP-2670).

Revendications pour l'Etat contractant suivant : ES

- 50 1. Procédé de production d'une substance polysaccharidique RON biologiquement active qui comprend la culture d'une souche de microorganismes choisie au sein du groupe constitué par les souches de *Leuconostoc mesenteroides*, sous-espèce *dextranicum*, BL-75 (FERM BP-2242), NCFB 517 (FERM BP-2711), NCFB 531 (FERM BP-2712), NCFB 861 (FERM BP-2713), NCFB 864 (FERM BP-2714), NCFB 880 (FERM BP-2715), 46-1 (FERM BP-2670) ATCC 1956 et IFO 3349 ainsi que leurs formes mutantes,
 - 55 pour produire dans un milieu de culture contenant du saccharose comme source de carbone de la substance polysaccharidique RON biologiquement active possédant les propriétés suivantes, et la récupération de la substance polysaccharidique RON biologiquement active à partir du milieu de culture:

- (1) Forme : la substance est une poudre amorphe de couleur blanche, inodore et sans saveur;
 - (2) Solubilité : la substance est hydrosoluble, mais elle prend un aspect blanc laiteux et visqueux lorsque la concentration est élevée; la substance est soluble dans le formamide et dans le diméthylsulfoxyde, mais elle est insoluble dans l'alcool, l'acétone, le benzène, l'acétate d'éthyle, l'hexane, le chloroforme et le tétrachlorure de carbone;
 - (3) pH de la solution aqueuse : la solution aqueuse est neutre ou légèrement acide;
 - (4) Constituant saccharidique : la substance est uniquement composée de glucose;
 - (5) Analyse élémentaire: la substance comprend 44,0% à 45,0% de C et 6,1 à 6,3% de H;
 - (6) Structure: la substance est un α -1,6-glycane qui contient une petite quantité de chaînes latérales ramifiées en 3,6;
 - (7) Protéines : la substance ne contient pratiquement pas de protéines;
 - (8) Poids moléculaire : la substance ne traverse pas une membrane de dialyse, son poids moléculaire est donc supposé être supérieur à 10.000 daltons;
 - (9) Rotation spécifique : $[\alpha]_D^{25} = +190^\circ - +220^\circ$ (C = 0,5, formamide);
 - (10) Réaction colorimétrique : la substance donne un résultat positif à la réaction avec l'anthrone-acide sulfurique et à la réaction avec le phénolacide sulfurique, mais un résultat négatif à la réaction du biuret, à la réaction de Lowry-Folin, à la réaction d'Elson-Morgan et à la réaction avec l'iode;
 - (11) Point de fusion: la substance n'a pas de point de fusion défini;
 - (12) Spectre d'absorption dans l'ultraviolet : la substance n'a pas d'absorption caractéristique;
 - (13) Spectre d'absorption dans l'infrarouge : la substance présente une absorption caractéristique de l' α -glycane;
 - (14) Spectre de RMN du ^{13}C : la substance montre les valeurs de transition chimique caractéristiques de l' α -1,6-glycane pour les principaux signaux et
 - (15) Activité biologique : la substance a une activité anti-tumorale.
2. Procédé selon la revendication 1 dans lequel on utilise la souche de *Leuconostoc mesenteroides*, sous-espèce *dextranicum* BL-75 (FERM BP-2242) et de la souche de *Leuconostoc mesenteroides*, sous-espèce *dextranicum*, 46-1 (FERM BP-2670) en tant que micro-organisme.

FIG. 1

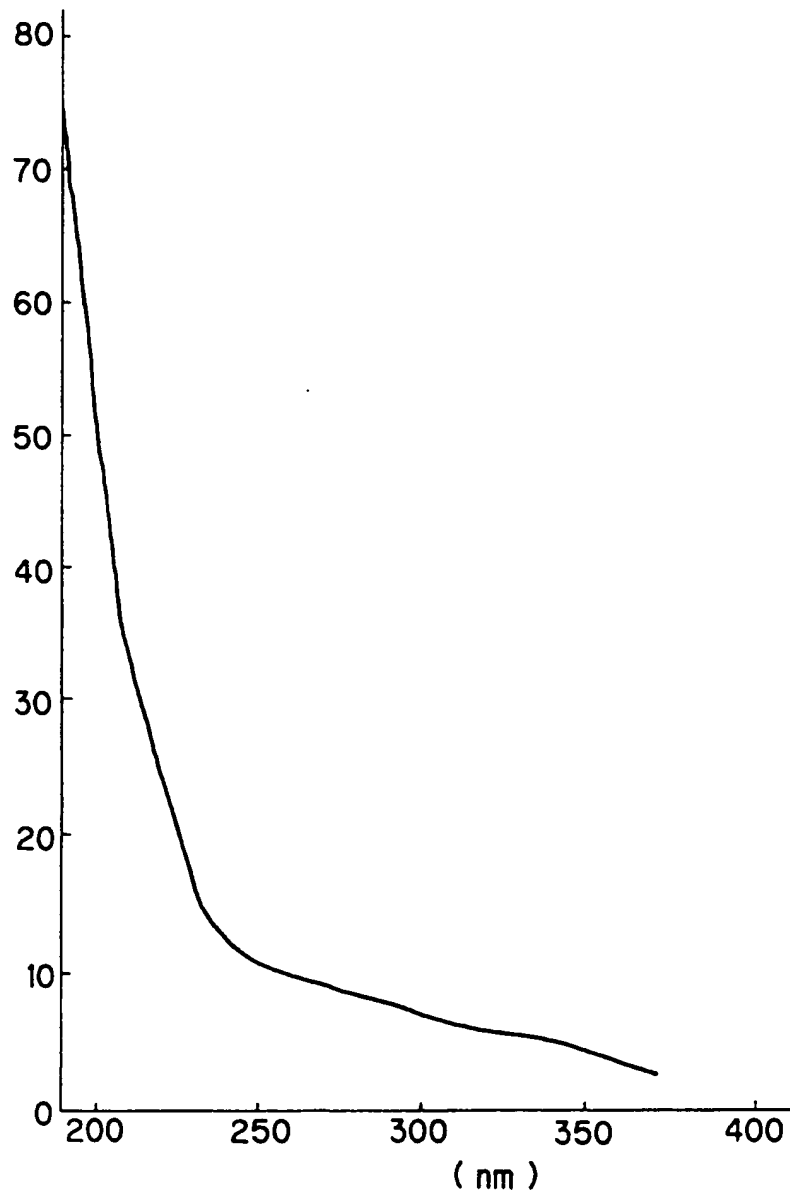


FIG. 2

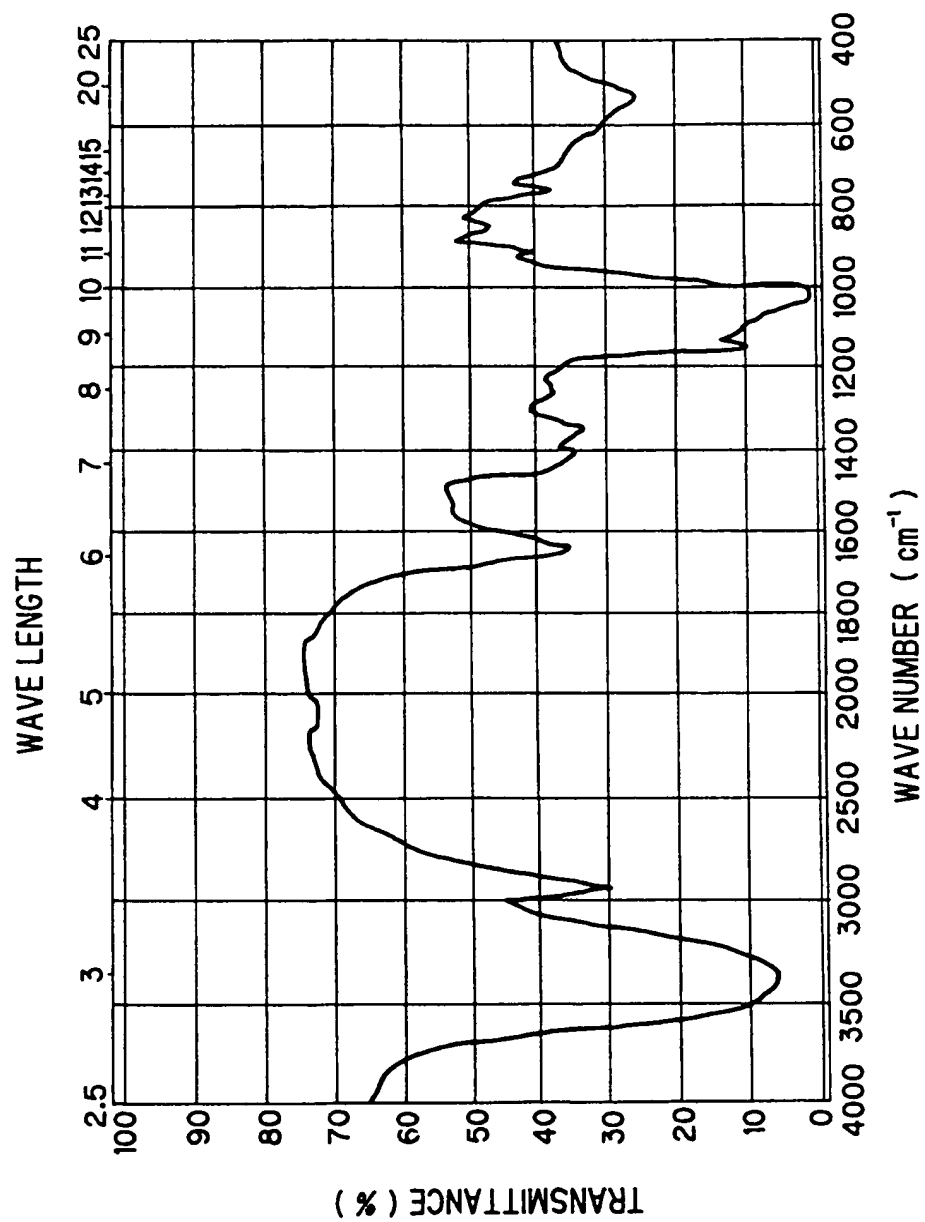


FIG. 3

